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Casini, Angela; Reedijk, Jan

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## MINIREVIEW

## Interactions of anticancer Pt compounds with proteins: an overlooked topic in medicinal inorganic chemistry?

Angela Casini<sup>\*a</sup> and Jan Reedijk<sup>bc</sup>

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A critical discussion is presented about the possible role of Pt–protein interactions in the mechanisms of action of platinum anticancer compounds. Although, since 40 years from its discovery, cisplatin and analogues are believed to exert their therapeutic effects *via* direct interactions with nucleic acids, several proteins/enzymes have recently appeared to be involved in the compounds' overall pharmacological and toxicological profiles, apart from classical serum transport proteins and metal detoxification systems. As an example, the emerging role of zinc finger proteins is noteworthy in the activity of platinum drugs. Moreover, the pursuit of novel platinum candidates that selectively target enzymes is now the subject of intense investigation in medicinal bioinorganic chemistry and chemical biology. An overview is presented of the most representative studies in the field, with particular focus on the characterization of the Pt–protein interactions at a molecular level, using different biophysical and analytical methods.

## 1. Introduction

Platinum coordination compounds, well known before Alfred Werner classified coordination compounds<sup>1</sup> and one of them, cisplatin, recognized as anticancer drug in the late 1960s,<sup>2–4</sup> have been studied intensely for several decades now. Most recently, the major aim to study these compounds stems from the wish to learn about their mechanisms of pharmacological action in the expectation to improve administration protocols and making new drugs. This work has been reviewed regularly,<sup>5–8</sup> including by one of us.<sup>9–11</sup> During the last four decades a strong focus has been on the irreversible (kinetically inert) binding of such platinum compounds to DNA, resulting in halting the cell divisions and leading to apoptosis. However, even from laboratory coordination chemistry experiments it is predictable that Pt-amine compounds would also react with proteins.

In this minireview we will focus on the latter biological targets and explore the evidences of Pt–protein binding relevant to the drug's mechanisms of action. Indeed, until 2006, only a limited number of biophysical studies have appeared dealing with the interactions of anticancer metallodrugs with proteins. These studies mostly concerned the two major serum proteins, albumin and transferrin, involved in the transport of metallodrugs, as well

as metallothioneins, small, cysteine-rich intracellular proteins, primarily involved in storage and detoxification of soft metal ions, including some early work by one of us.<sup>12–15</sup>

Subsequently, the general consensus on the crucial role of the interactions of metallodrugs with protein targets in determining the compounds' pharmacological action, uptake and bio-distribution, as well as their overall toxicity profile, resulted the number of studies to increase exponentially.<sup>16,17</sup> Nowadays, cisplatin and related compounds are known to bind to several classes of proteins with different roles, including transporters, antioxidants, electron transfer proteins, DNA-repair proteins, as well as proteins/peptides simply used as model systems to characterize the reactivity of metallodrugs *in vitro*, but that are also present *in vivo*. Recently, a study by Bischin *et al.*<sup>18</sup> has reviewed the Pt-species bound to proteins; in fact, many of the reported crystallographic structures are also available in the Heavy Atom Database (HAD) in relation to the heavy atom replacement methodology, and not always refer to Pt-based anticancer compounds.

Below, we will discuss in detail about the various proteins/enzymes that have been studied so far and that have been shown to interact with platinum anticancer drugs. We shall also comment on how the investigational approach has evolved in this field from studies involving simple amino-acid models to model peptides and proteins, or even protein mixtures/cell extracts. Attention will be paid to those proteins that influence the mechanisms of action of platinum drugs at different levels, from contributing to metal transport and uptake, to being real pharmacological targets. For proper understanding, a brief description of the novel techniques and methodologies to characterize such metal–proteins interactions is presented.

<sup>a</sup>Pharmacokinetics, Toxicology and Targeting, Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: a.casini@rug.nl; Tel: +31 50 363 8006

<sup>b</sup>Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

<sup>c</sup>Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, 11451 Riyadh, Saudi Arabia

## 2. Anticancer platinum compounds and initial studies of their interactions with proteins

The success of cisplatin (*cis*-diamminedichloridoplatinum(II); *cis*-Pt(NH<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub>) in the clinic has resulted in numerous derivatives as second- and third-generation drugs (see Fig. 1 for a selection). Any chemotherapeutic treatment of cancers, worldwide, is currently done with a combination of anticancer drugs, to reduce the toxic side effect of single compounds. In fact, 50% of all these combinations nowadays contain a platinum drug. In more recent years, we and others have been looking at more complicated compounds, such as dinuclear, trinuclear and even mixed-metal heteronuclear Pt(II), and even Pt(IV) compounds have been the subject of investigation.<sup>10,11,19</sup>

In Fig. 1 we have included a selection of the most recently synthesized drugs, based on improved kinetics (picoplatin),<sup>20</sup> oral administration (satraplatin),<sup>21</sup> dinuclear azolate bridged compounds,<sup>22,23</sup> trinuclear diamine-bridged species,<sup>24</sup> and a group of compounds where a tail on the side arm can contain either another metal such as Cu,<sup>25</sup> or Ru,<sup>19,26</sup> or an intercalator.<sup>10,27</sup> The reader should realize that there are many more examples and for details we refer to reviews of others and us.<sup>28–32</sup>

The design of these compounds has in most – if not all – cases been based on their (improved) binding to DNA, in particular at guanines. However, as previously mentioned, important findings have been reported in the literature revealing that metal-based drugs of different families, including Pt complexes, can target proteins and enzymes *in vitro*.<sup>33</sup> Moreover, direct interactions with proteins have been demonstrated to take active roles in the mechanisms of action of cisplatin, either affecting the sensitivity of certain cancer cells to platinum-induced DNA damage (as reported for the high-mobility group proteins (HMG)),<sup>34</sup> or in the development of cancer cell resistance to Pt treatment.<sup>35</sup> In 2008 Davey *et al.* reported an X-ray crystallographic and biochemical investigation of the reaction of cisplatin and oxaliplatin with nucleosome core particle and naked DNA, revealing that histone octamer association can modulate DNA

platination.<sup>36</sup> Platinum adduct formation was found to occur not only at DNA sites, but also at specific histone methionine residues, which could serve as a nuclear platinum reservoir influencing adduct transfer to DNA.

Within this frame, HSAB (hard soft acid base) theory predicts that cisplatin, being a soft metal, has a high affinity to sulfur-containing ligands. Indeed, sulfur-containing proteins and peptides do play a significant role in the functioning of platinum drugs, because of their high affinity for platinum, their abundance (*e.g.* albumin), and their involvement in metal-ion transport. It is unclear how platinum coordination compounds can generate DNA adducts with so many competing sulfur-donor ligands in the cell.<sup>37</sup> Initially, model amino acids have been used as representative systems to characterize the mechanisms of Pt–protein binding. These first experiments improved the understanding of the metabolite form, by which the metal complex enters the tumour cells, and whether and how this metabolized complex is already inactivated at this time.<sup>38</sup> Thus, efficient analytical speciation methods were introduced into this important field of research to investigate the mechanisms of action of Pt-chemotherapeutics, mostly based on various HPLC separation techniques hyphenated to sensitive and selective detectors. In this context inductively coupled plasma mass spectrometry (ICP-MS) has been crucial due to the relative simplicity in measuring online signals of Pt-containing species (hydrolysis products and metabolites) from anticancer drugs. The findings from HPLC-ICP-MS have been further complemented by structural information, for example by electrospray ionization mass spectrometry (ESI-MS). In order to elucidate rapid kinetic changes even capillary electrophoresis (CE) techniques were employed for fast separation of Pt species.<sup>39</sup>

Using the above-mentioned techniques it was shown that DNA is the thermodynamically favoured bound end product, whereas the platinum-containing agents react more rapidly with other ligands, such as thiolates and thioethers.<sup>37</sup> Several studies have been reported in the 1990s; to be mentioned are the work of Sadler and co-workers on the activation of cisplatin towards

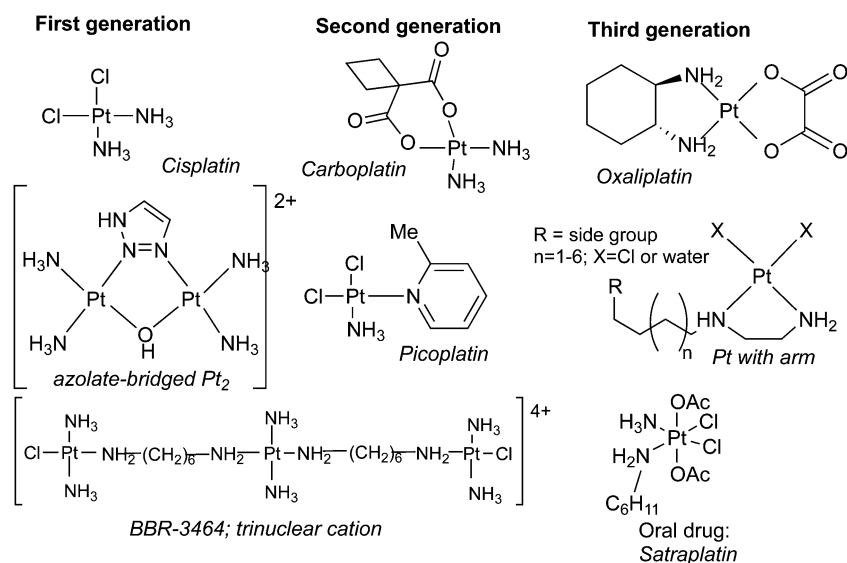


Fig. 1 Clinically used platinum anticancer compounds and a selection of active derivatives.

nucleoside binding upon reaction with Met residues,<sup>40,41</sup> and the results of Lempers *et al.* on binding to proteins and GSH.<sup>12–14</sup> Studies on methionine and other sulfur donors suggest that platinum species may migrate, after initial binding to sulfur-containing ligands, to DNA *in vivo*.<sup>37</sup> As such, because of their ubiquity in the cell nucleus, the histone methionine residues mentioned above could serve as an important pool for transfer of Pt adducts to the DNA.

Concerning metallodrug–intact protein interactions, a series of pioneering studies based on ESI-MS, and carried out during the 1990s and early 2000s, highlighted the advantages of this method and defined the experimental conditions for its application to simple metallodrug–protein samples. Most of these studies focused on the reactivity of cisplatin and analogues with ubiquitin (Ub), taken as the reference model protein, and in some cases aimed at determining the competitive binding of platinum complexes with other biological nucleophiles.<sup>42–45</sup> Interactions of Pt(II) drugs with other model proteins (cytochrome c, superoxide dismutase, lysozyme, myoglobin), or mixtures of them, were further investigated, also by one of us, by various techniques including MS, NMR, ICP-OES and X-ray crystallography to provide a description of the system at a molecular level.<sup>46–54</sup> Fig. 2 depicts the global X-ray structure of cisplatin bound to hen egg white lysozyme (HEWL, PDB 2I67).

In general, the obtained results implied that platinum drugs dissolved in biological media – thus, in the presence of many chemical components, including macromolecules – can manifest a chemical reactivity that is profoundly distinct from that observed when they are just dissolved in simple buffered solutions. These observations posed important “caveats” for researchers to extrapolate the behaviour observed in solution for metallodrugs to that believed to occur inside cells.

Similar studies on the interactions of platinum compounds with hemoglobin (Hb) were performed to provide evidence that binding of platinum to Hb might lead to side effects *in vivo*. Hb is a globular tetrameric protein consisting of four subunits held together through non-covalent interactions, each with a  $M_w$  of about 15–16 kDa. Each protein subunit is an individual molecule that joins to its neighbouring subunits through intermolecular

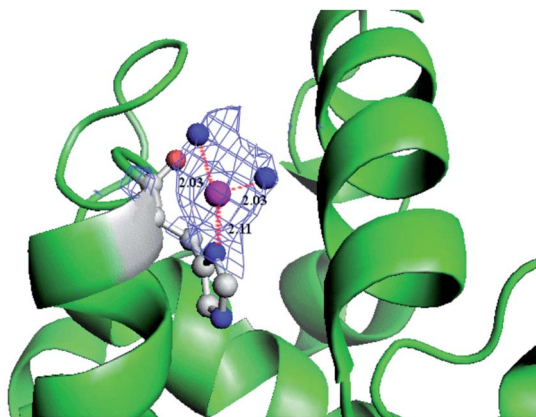
interactions and carries a heme group having Fe as the central atom. Mass spectrometry studies proved that platinum drugs bind Hb at clinically relevant concentrations, while heme release was also observed upon Pt binding.<sup>55</sup> Moreover, cysteine and proline residues were shown to be involved in the Hb–Pt interaction. In the case of oxaliplatin, MS/MS identification of the parent oxaliplatin and the Pt(dach) [dach = 1,2-diaminocyclohexane] moiety in the Hb adducts supports the hypothesis that an extensive amount of oxaliplatin bound to the Hb in erythrocytes may lead to a reduced dose of oxaliplatin available for DNA binding.

Interestingly, the activation of the Pt(IV) compound satraplatin by haemoglobin, cytochrome c and liver microsomes was observed *in vitro* combining HPLC-ICP-MS and visible absorption spectrophotometry, suggesting a possible role of metal-containing redox proteins acting as reducing agents for the Pt centre.<sup>56</sup>

It must be noted that thanks to the fast development of high-resolution analytical methods in recent years, it has been possible to re-analyse and further characterize some of the above mentioned Pt–protein samples. As an example, the interaction between oxaliplatin and the model protein Ub was investigated in a top-down approach by means of high-resolution ESI-MS, using diverse tandem mass spectrometric (MS/MS) techniques, including collision-induced dissociation (CID), higher-energy C-trap dissociation (HCD), and electron transfer dissociation (ETD).<sup>57</sup> Similarly, high-resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT ICR-MS) studies provided further evidence for cisplatin as a protein cross-linking reagent.<sup>58,59</sup> In some cases, comparison of different ionization techniques, *i.e.* matrix-assisted laser desorption/ionization (MALDI) and nanoelectrospray ionization mass spectrometry (nESI-MS), for the analysis of small protein–Pt anticancer drug interactions, was possible.<sup>60</sup>

In very recent years, more complex systems have been analysed and examples of *metallomics* strategies based on different techniques (*e.g.* NMR,<sup>61</sup> fluorescence, MS or X-ray absorption spectroscopy (XAS)), and applied either on cell extracts or directly on cells, appeared in the literature, mainly aiming at determining biological targets, metallodrug distribution, as well as metal speciation.<sup>62,63</sup> For example, interesting information on the cellular targets of cisplatin came from a study where *E. coli* cells were treated with the drug, and multidimensional liquid chromatography and electrospray ionization tandem mass spectrometry were used to identify as many as 31 proteins to which platinum was bound.<sup>64</sup> These included high abundance enzymes and ribosomal proteins, as well as DNA- and RNA-binding proteins. One of the most interesting results of that study was that, contrary to what one would expect from HSAB theory, according to which both Cys and Met would be the primary binding sites for cisplatin on proteins, carboxylate and hydroxyl groups were identified as the platinum coordination sites in 18 out of 31 proteins and Met was identified as the binding site only 9 times, while no binding to cysteine was reported.

In other studies, Hambley *et al.* showed that X-ray absorption near edge spectroscopy (XANES) can be used to identify the distribution and oxidation states of Pt(II) and Pt(IV) in cells and in spheroids.<sup>65,66</sup> [<sup>1</sup>H, <sup>15</sup>N] HSQC NMR spectroscopy was also successfully used to monitor the stability of <sup>15</sup>N-labelled cisplatin



**Fig. 2**  $2F_o - F_c$  map at  $1\sigma$  covering cisplatin/HEWL adduct, covering platinum(II) (magenta) that interacts with Ne of His 15 and with two ammonia ligands (blue) with the relative bond lengths (Å) indicated. Reproduced using the coordinates of ref. 47.



in aqueous extracts of cancer cells. Interestingly, from these latter studies glutathione (GSH) appeared not to be the major target of cisplatin in the cytoplasm.<sup>67</sup>

Monitoring biotransformation products of platinum drugs in tissue is certainly an advance investigation in medicinal inorganic chemistry. Esteban-Fernandez *et al.* performed *in vivo* studies by administering platinum drugs to experimental rats and examining Pt–protein binding in the kidney and the inner ear by using 2D liquid chromatography coupled to ICP-MS to elucidate the severe induced nephrotoxicity and ototoxicity.<sup>68</sup> The results clearly indicated that not only the total Pt content, but rather the actual Pt-containing species are the responsible factor for the alteration of organ functions. Moreover, speciation studies revealed complete Pt binding to proteins. More recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) imaging was applied to study the spatial distribution of oxaliplatin in tissue sections of rat kidneys.<sup>69</sup> The obtained results showed that oxaliplatin was localized at the periphery of the kidney, suggesting that the drug cannot penetrate deeply into the organ, thereby explaining its reduced nephrotoxicity in comparison to cisplatin.

### 3. Proteins involved in the transport and resistance mechanisms of platinum drugs

#### 3.1 Introduction

Although most patients initially do respond well to platinum-based chemotherapy, a considerable percentage eventually develop drug resistance and relapse. Platinum resistance is considered multi-factorial and includes both mechanisms that limit the formation of platinum–DNA adducts, as well as mechanisms that prevent cell death following drug-induced damage.<sup>70–74</sup> Among the mechanisms limiting Pt–DNA adduct formation the following two have been recognized: (i) impaired transport leading to reduced platinum accumulation;<sup>75</sup> and (ii) inactivation of platinum compounds by sulfur-containing molecules. In both cases the direct interaction of Pt drugs with proteins plays a major role, as will be discussed below.

Reduced cellular accumulation of platinum either by impaired uptake or increased efflux is often found in cells selected for cisplatin resistance, both *in vivo* and *in vitro*, and is generally considered as one of the most consistent characteristics of platinum resistant cells.<sup>75</sup> Facilitated or active transport systems, as well as passive diffusion, are both relevant for the cellular uptake of platinum drugs.<sup>76,77</sup> In addition, general drug uptake/efflux systems in the intestine, liver and kidney are increasingly found to be important and may have a major impact on drug disposition and response to platinum-based chemotherapy.<sup>78</sup>

Concerning transport mechanisms for Pt drugs, a series of experimental evidences, well reviewed by Hall *et al.*,<sup>79</sup> leads to conclude that cisplatin most likely enters the cell *via* two pathways: (a) passive diffusion and (b) facilitated and active uptake by a number of transport proteins, like CTR1.<sup>80</sup> Initially, passive diffusion through the cellular lipid bilayer was considered to be the dominant process involved in drug uptake and distribution. However, more recently the concept of carrier mediated and active uptake of commonly prescribed drugs has become the rule rather than the exception.<sup>81</sup> Thus, membrane transporters and

channels, collectively known as the *transportome*, are increasingly recognized as important determinants of tumour cell chemosensitivity and chemoresistance. Membrane transporters of platinum-based anticancer agents determining active platinum uptake and efflux pathways, as well as their clinical significance have recently been reviewed by Burger *et al.*,<sup>82</sup> including organic cation transporters (OCTs) belonging to the SLC22 subfamily, solute carriers (SLCs) and ATP-binding cassette (ABC) multi-drug transporters. Here, we aim at recalling only those that have been most studied and characterized by both pharmacological and biophysical methods, namely the copper influx transporters (CTRs), as well as the copper chaperon protein Atox1. In addition, a paragraph on serum proteins albumin and transferrin as possible platinum transport systems will be also included.

Concerning the resistance mechanisms to Pt drugs we will primarily summarise and discuss the studies on metallothioneins (MTs), even though several other proteins/peptides are also involved.<sup>35</sup> For example, increased levels of both P-glycoprotein (Pgp) and glutathione-S-transferases have been correlated to drug resistance in certain tumours and inhibitors of both proteins have been used in combination therapies with, for instance, cisplatin.

#### 3.2 Copper transporters and chaperons

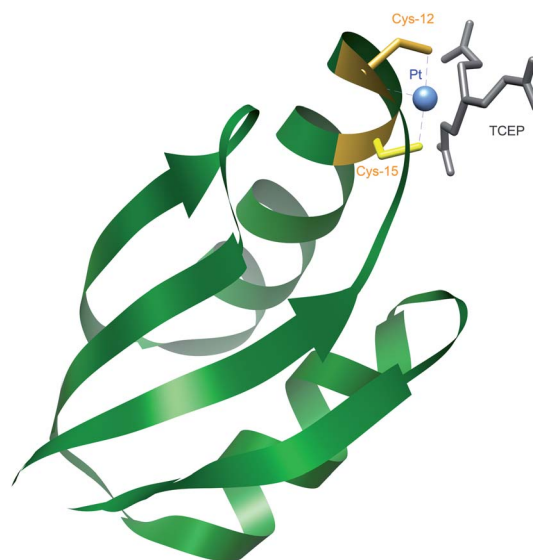
Copper transporters have been proposed to be involved in cellular import and export of platinating agents, as well as in cisplatin resistance mechanisms.<sup>80,83</sup> In particular, expression of the human copper transporter 1 (hCTR1) is thought to result in increased sensitivity to cisplatin, whereas expression of two Cu(I) proteins exporting ATPase, *i.e.* ATP7A and ATP7B, is believed to be involved in the resistance to cisplatin, either by sequestering drug away from its targets (ATP7A), or by exporting the drug from the cell (ATP7B). CTR1 (copper transporter 1, SLC31A1) is an evolutionarily conserved copper influx transporter present in plants, yeast, and mammals, and is the main copper importer in mammalian cells. The human version, hCTR1, is expressed in all tissues and is a key player in the homeostatic regulation of intracellular copper levels, to ensure that nutritional delivery of copper to enzymes, such as cytosolic Cu,Zn-superoxide dismutase. hCTR1 is located in the plasma membrane and is constituted by three transmembrane helices, an extracellular N-terminal domain and a cytosolic C-terminal domain.<sup>84</sup> Three hCTR1 molecules form a symmetric trimer with a channel-like architecture, as revealed by electron microscopy. Interestingly, methionine (Met)-rich motifs located in the N-terminal domain and in the inner side of the channel pore are critical for the binding of copper.<sup>85</sup>

hCTR1 has been shown to play an essential role in the cytotoxic effects of platinum drugs in cancer cells.<sup>86</sup> Moreover, cisplatin treatment of a cell line expressing hCTR1 revealed the time- and concentration-dependent appearance of a stable hCTR1 multimeric complex, consistent with a homotrimer, which was not observed following copper treatment of these same cells. Mutagenesis studies identified two methionine-rich clusters in the extracellular amino-terminal region of hCTR1 that were required for stabilization of the hCTR1 multimer by cisplatin, suggesting that these sequences bind cisplatin and, subsequently, form crosslinks between hCTR1 polypeptides.<sup>87</sup>

Natile and co-workers investigated the binding of platinum complexes to the Met-rich domain by different techniques including mass spectrometry and NMR spectroscopy.<sup>88</sup> According to their findings, cisplatin appears to easily form adducts with the peptide domain, in which all the original ligands of Pt are lost and replaced by the S-donor Met groups. On the basis of these observations cisplatin would be actually sequestered by hCTR1 and not transported, while a possible transport system could be actually an endocytotic process, incorporating a portion of the extracellular milieu (containing non-degraded cisplatin) into vesicles, which are subsequently delivered to subcellular compartments. These latter results might be in accordance with a more recent paper reporting on the fact that overexpression of hCTR1 in the human embryonic kidney (HEK) cell line did not result in increased sensitivity to cisplatin.<sup>89</sup> Other studies based on MS and NMR spectroscopy characterized the binding of platinum compounds with synthetic peptides corresponding to hCTR1 Met motifs at a molecular level, in some cases highlighting the differences among the various Pt drugs.<sup>90,91</sup>

Once platinum enters the cells, although many different systems appear implicated in cisplatin trafficking, mounting evidence suggests a linkage between cisplatin resistance and the human copper homeostatic proteins Atox1 and ATP7A or ATP7B.<sup>92</sup> The copper chaperone Atox1 binds Cu(I) at a conserved CXXC motif and delivers it to the N-terminal metal binding domains (MBDs) of ATP7B and ATP7A, which are Cu(I) specific P1B-type ATPases. Each human Cu(I) ATPase has six MBDs, which also bind Cu(I) with CXXC motifs and resemble Atox1 in the overall structure.<sup>93</sup> The structure of a stoichiometric cisplatin–Atox1 adduct (Pt–Atox1) was determined at 1.6 Å resolution showing a Pt(II) ion coordinated to Cys12 and Cys15 from the CXXC motif.<sup>94</sup> The geometry is square planar with the two cysteine ligands oriented *trans* to one another. The remaining ligands are provided by the backbone amide nitrogen of Cys12 and an exogenous donor best modelled as a 2-carboxyethylphosphane (TCEP) molecule with a TCEP(P)–Pt distance of 2.48 Å (Fig. 3). In the same paper the structure of a dimeric cisplatin adduct Pt–(Atox1)<sub>2</sub> was also reported at 2.14 Å resolution. Overall, the two structures support the idea that the cisplatin interaction with Cu(I) binding motifs leads to unfavourable therapeutic outcomes, not only due to unproductive cisplatin trafficking, but perhaps also as a result of aberrant Cu(I) transport in cisplatin resistant tumours.

Using solution and in-cell NMR spectroscopy to probe the interaction of cisplatin with the human Atox1 in a physiological environment, Arnesano *et al.* showed that Pt binding to the Cu chaperone follows at least two steps: initially a Pt–Atox1 adduct is formed, while at longer reaction times protein dimerization and loss of the amines from cisplatin are observed.<sup>95</sup> Such a process is reminiscent of the copper-promoted formation of Atox1 dimers which have been proposed to be able to cross the nuclear membrane and act as a transcription factor. In the same paper it was reported that overexpression of Atox1 in *E. coli* reduces the amount of DNA platination and, consequently, the degree of cell filamentation, suggesting a possible mechanism of resistance to cisplatin chemotherapy associated to overexpression of Cu transporters in cancer cells. Finally, it is worth mentioning that other recent NMR studies described Atox1 unfolding induced by cisplatin.<sup>96</sup>



**Fig. 3** Representation of the crystal structure of cisplatin bound to an Atox-1 monomer (PDB code 3IWL).

### 3.3 Serum proteins

Upon intravenous application reactions of cisplatin with human serum proteins, both albumin (HSA) and transferrin (Tf) are thought to play an important role in the metabolism of this anticancer drug. HSA is the most abundant protein (about 52% of total serum protein) with a concentration of 40–45 g L<sup>−1</sup> in healthy humans (*ca.* 600 μM; *M<sub>w</sub>* 66–67 kDa). It comprises a single chain of 585 amino acids organized in three similar domains, each composed of two subdomains.<sup>97</sup> HSA is known to bind a remarkably wide range of drugs, thereby restricting their free, active concentrations.<sup>98</sup> Reducing this binding affinity represents a major challenge in drug development. However, HSA can also be exploited for targeted delivery strategies to develop compounds that can selectively accumulate in tumour cells, including platinum complexes.<sup>99,100</sup>

Concerning platinum drug binding to HSA several reports have appeared in the literature; however, still conflicting information is available concerning the actual Pt preferential binding sites on HSA. According to NMR spectroscopic studies, a Met298 *S,N*-macrochelate is a major cisplatin binding site, and monofunctional adducts involving Cys34 and surface histidine residues are also known to be formed.<sup>101</sup> Multidimensional liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of serum samples treated with cisplatin for 3 h identified a total of five specific binding sites of Pt for HSA, including the cysteine residue Cys34, two methionine sites (Met329, Met548) and the tyrosine and aspartate O-donor sites Tyr150 (or Tyr148) and Asp375 (or Glu376).<sup>102</sup> Similarly, when CE was applied to assess cisplatin interactions with HSA, strong metal–protein coordination appeared to occur at several HSA sites.<sup>103</sup> Recently, the combination of tryptic digestion with LC-MS/MS analysis has provided additional information revealing that cisplatin can crosslink His67 and His247 at the interface between domains I and II, that are part of the major zinc binding site on albumin.<sup>104</sup>

The interactions of polynuclear platinum complexes analogues of the trinuclear BBR3464 (Fig. 1) with HSA were also studied by various techniques. Evidence for pre-association, presumably through electrostatic and hydrogen-bonding, was obtained from fluorescence and circular dichroism spectroscopy and ESI-MS. When such compounds contain Pt–Cl bonds, further reaction will take place presumably through displacement by sulfur nucleophiles. The interaction, but not deactivation, of the reported Pt complexes on HSA suggests a new and promising pathway for a polynuclear platinum drug.<sup>105</sup>

To study the carboplatin–serum proteins interaction, a sensitive method using size exclusion chromatography coupled to inductively coupled plasma mass spectrometry (SEC-ICP-MS) was developed.<sup>106</sup> The results showed that carboplatin–albumin and carboplatin–globulin complexes were formed after the infusion of carboplatin, and the concentration of all platinum species decreased as the species were metabolized and continuously excreted from the human body. Besides the primary one-to-one binding of Pt to proteins, also aggregation of proteins was observed. This aggregation may result either from the cross-linking of proteins through platinum, or from an association facilitated by platinum. It is worth mentioning that Szpunar *et al.* were the first to use hyphenated speciation techniques with SEC-ICP-MS to investigate interaction of cisplatin with serum.<sup>107</sup> Afterwards, other groups approached the problem of platinum drugs quantification in serum and plasma. These latter studies have been recently reviewed by Michalke,<sup>38</sup> and we refer the reader to that paper for deeper insight in the methodology.

Human serum transferrin (Tf) is a single-chain glycoprotein containing 679 amino acids with a molecular mass of about 80 kDa and is found in blood at a concentration of about  $2.5 \text{ g L}^{-1}$  (35  $\mu\text{M}$ ). Tf acts as an iron transporter and is capable of binding two iron(III) ions ( $\text{Fe}^{3+}$  is bound selectively over  $\text{Fe}^{2+}$ ).<sup>108</sup> Tf is normally only 30% saturated with iron in the body, and at least 30 other metal ions can also bind to Tf.<sup>109</sup> Therefore, it is possible to use Tf as a metal transporter within the body and the cellular uptake mechanism *via* the Tf-transferrin receptor transport system has the potential to be exploited for site-specific delivery of various therapeutic metal ions, drugs, proteins and genes.<sup>110</sup> In the case of metallodrugs the resulting Tf-conjugates may significantly improve the cytotoxicity and selectivity of the drugs itself. Thus, ESI coupled to Q-TOF mass analyser has been widely used to characterise the interaction of cisplatin and oxaliplatin with human serum transferrin, and allowed to determine the binding of cisplatin to a specific threonine residue.<sup>111–113</sup> It is worth mentioning that previously reported NMR studies by Cox *et al.* revealed Pt binding to several Met residues of Tf.<sup>114</sup>

Recently, one of us developed a method based on the coupling of high-resolution size-exclusion liquid chromatography (SEC), using a polymer stationary phase with ICP-MS. This approach was applied to study the interactions of cisplatin with the serum proteins albumin and transferrin.<sup>115</sup> Metal binding was found to be dependent on the protein concentration and on the incubation time of the sample. Cisplatin was found to be moderately reactive towards the proteins without any discrimination/selectivity, in accordance with the results obtained by an ESI-MS based approach to study drug binding to protein mixtures.<sup>49</sup> Moreover, cisplatin binding to a cysteine residue in a model peptide resembling the Tf binding pocket for Fe ions

(<sup>239</sup>KDCHLAQVPSHTV<sup>251</sup>), appeared to be the most favoured according to MS data, although it is worth mentioning that the Cys residue is involved in a disulfide bridge in the native protein.

### 3.4 Metallothioneins

Human metallothioneins (MTs) are a class of small (*ca.* 7000 Da) cysteine- and metal-rich proteins, abundant in most human tissues. They are represented by four highly conserved isoforms (MT-1/-2/-3/-4). MT-1 and MT-2 occur ubiquitously in high amounts in mammalian cells, and in contrast to MT-3/-4, the biosynthesis of MT-1/-2 is induced by a variety of compounds, including hormones, cytokines and metal compounds, such as cisplatin.<sup>116,117</sup> Human MTs are composed of a single polypeptide chain of 61–68 amino acids including 20 cysteines. The cysteine thiolates are involved in the binding of up to seven divalent metal ions, forming two independent metal–thiolate clusters in which each metal is tetrahedrally coordinated by both terminal and bridging thiolate ligands.<sup>118–120</sup> Naturally occurring MTs usually contain seven Zn(II) ions. However, these metals can be displaced by other metal ions *in vivo* that have a higher affinity for thiolates such as Cd(II), Hg(II) and Pt(II), and the molecular mechanisms of the Pt(II) interactions with MT-1/-2 have been the subject of numerous studies.<sup>121</sup> Of note, the reactions of human Zn<sub>7</sub>MT-2 with twelve *cis/trans*-[Pt(N-donor)<sub>2</sub>Cl<sub>2</sub>] compounds, including new generation drugs, were investigated by ESI-MS and the Pt-adducts characterized. A comparison of reaction kinetics revealed that *trans*-Pt(II) compounds do react faster with Zn<sub>7</sub>MT-2, replacing zinc, than *cis*-Pt(II) compounds do.<sup>122</sup> The characterization of the products showed that, while all ligands in *cis* compounds were replaced by cysteine thiolates, the *trans* isomers retained their N-donor ligands, thus remaining in a potentially active form. Recently, one of us studied competitive binding of cisplatin towards a mixture of ubiquitin and MT-2 by ESI-MS, showing that the drug has poor selectivity towards the selected proteins and can form adducts with both peptides.<sup>123</sup>

## 4. Possible targets: zinc-finger proteins

Several protein systems and enzymes need zinc ions to perform their biological function. Classification of zinc sites in proteins is generally into two categories: (i) *catalytic* sites with the presence of a readily exchangeable water ligand coordinated to the zinc (*i.e.* hydrolases) and (ii) *structural* sites with no coordinated water and only protein residues in the coordination sphere. This “coordinative saturation” has as its purpose the creation or maintenance of an appropriate secondary/tertiary structure in the protein (*i.e.* zinc fingers or superoxide dismutase).<sup>124</sup>

Structural zinc covers an important area and is described by ligation of four protein-derived histidine and/or cysteine ligands yielding a coordinatively saturated central metal ion, and prominent amongst these sites are the zinc fingers. From the human genome sequencing project, it has become apparent that the zinc-finger proteins themselves constitute 2–3% of the entire human genome, and are the most common DNA binding motifs found in human transcription factors.<sup>125,126</sup> The diverse range of biological functions of zinc-fingers and zinc-finger-like proteins includes DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and

assembly, contributing to the manifestation of fundamental cellular processes such as development, differentiation, and tumour suppression. The zinc finger (ZF) motif, first described in the transcription factor TFIIIA from the clawed toad *Xenopus laevis*,<sup>127</sup> exhibits a notably diverse array of structure and functions, the latter involving important cellular processes such as transcription, DNA repair, cellular signalling, metabolism and apoptosis. It is worth mentioning that, typically, the term ZF implies a definite number of amino-acid residues within the protein, usually 30 to 40, with suitable metal-binding sites composed of cysteines (Cys; monodentate or bridging) and histidines (His). A key component of this system is the zinc ion ( $\text{Zn}^{2+}$ ), which binds to the residues in a tetrahedral-based environment providing essential elements of the structure. Release or substitution of the central zinc ion, as well as mutation of coordinating residues can result in a loss or impairment of the biological function. For their essential multiple biological roles ZF damage by oxidizing agents or redox-active metals has been regarded as a novel mechanism of carcinogenesis.<sup>128</sup> Interestingly, the zinc-finger coordination environments are susceptible to displacement of  $\text{Zn}^{2+}$  by other inorganic and transition metal ions, which therefore are able to alter the zinc-finger domain biological function.<sup>129</sup>

Within this frame, anticancer metal complexes have been reported to efficiently interact with zinc-finger proteins and enzymes, and, therefore, can be considered as possible medicinal targets for this class of drugs. As an example, one of us recently described the poly(adenosine diphosphate (ADP)-ribose) polymerase 1 (PARP-1) inhibition properties of different metal compounds including cisplatin.<sup>130</sup> PARPs are essential proteins involved in cancer resistance to chemotherapies. PARPs play a key role in DNA repair by detecting DNA strand breaks and catalysing poly(ADP-ribosylation),<sup>131</sup> and consequently, PARPs have been referred to as “the guardian angels” of DNA.<sup>132</sup> Notably, PARP-1, the most studied member of the PARP family, is characterized by the presence of two long zinc fingers (ZF-PARPs, also termed as nick-sensors), that are positioned upstream of the catalytic domain,<sup>133</sup> and mediate specific nicked DNA recognition.<sup>134</sup> PARP has also been shown to bind to platinum-modified DNA,<sup>135,136</sup> and a systematic *in vitro* study was recently conducted in which the effect of PARP inhibition on the ability of nuclear proteins to bind platinum-modified DNA was evaluated by photo-cross-linking experiments.<sup>137</sup> According to these results the activity of PARP, following exposure to platinated DNA, resulted in the dissociation of DNA-bound proteins. Moreover, PARP inhibitors were able to sensitize some, but not all, of the cell lines towards cisplatin. Other studies describe the binding of PARP-1 to platinum 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand cross-links on duplex DNA<sup>137,138</sup> and a very recent report<sup>139</sup> demonstrated that PARP-1 differentiates between normal and platinum-damaged DNA, having higher binding affinity for the cisplatin 1,2-d(GpG) cross-links than for the unplatinated DNA or other types of cisplatin–DNA cross-links.<sup>139</sup> In this latter study it was also shown that PARP-1 may shield the DNA lesion from repair and trigger a cytotoxic response. Overall, despite these numerous studies, the activity of PARP upon cisplatin treatment remains controversial and not fully understood.

As mentioned above, transcription factors are another important class of zinc-finger proteins. In this regard, it has been

shown that human DNA polymerase- $\alpha$  is inhibited by cisplatin *via* coordination with the cysteine residues on the protein's C4-ZF motif.<sup>140</sup> Ralph *et al.* have also shown by an ESI-MS approach that platinum compounds can interfere with binding of the transcription factor PU.1-DBD to a dsDNA molecule containing its consensus-binding site.<sup>141</sup> Moreover, cisplatin has also been recently reported to affect the conformation of the apo-form of the breast cancer susceptibility protein 1 (BRCA1) RING finger domain forming intra- and intermolecular Pt-BRCA1 adducts, where a preferential platinum-binding site was found at His117.<sup>142</sup> The same authors investigated the functional consequences of the *in vitro* platination of the BRCA1 RING domain by cisplatin and analogues, which resulted in the inhibition of the ubiquitin ligase activity of BRCA1.<sup>143</sup>

Of note, platinum(II) complexes have been reported to interact with the C-terminal finger of the HIV nucleocapsid NCp7 zinc finger leading to zinc ejection.<sup>144</sup> These latter studies show the opportunity of exploiting metal-based drugs as new classes of anti-HIV agents based on inhibition of HIV NCp7 function. Recently, the same authors showed that a platinated single-stranded oligonucleotide can alter the structure of a model ZF peptide and characterized this interaction at a molecular level by NMR spectroscopy.<sup>145</sup> Most importantly, these results have shown that the extent and rate of zinc displacement by inorganic compounds can be modulated by the nature (metal, ligands) of the reacting compound, and that DNA-tethered coordination complexes may be designed to target specific ZF motifs.

## 5. Perspectives for new drug design and targeted therapies

The knowledge of the possible reactivity of platinum complexes with peptides and proteins is essential also for the development of new targeted therapies for anticancer metallodrugs. From recent years, among the most representative examples, we could list Pt–HSA conjugates<sup>146</sup> that exploit endogenous albumin as drug carrier, since albumin is known to accumulate in solid tumours *via* the enhanced permeability and retention (EPR) effect; the latter being due to the pathophysiology of tumour tissues, characterized by angiogenesis, hypervascularity, a defective vascular architecture, and an impaired lymphatic drainage.

Modifications of the platinum carrier ligands and, to a lesser extent, of the leaving groups have also been widely exploited for achieving tumour tissue specificity. Among the possible ligands several examples include the use of peptidic moieties to facilitate platinum(II) uptake by peptide receptors.<sup>147–150</sup> Several Pt(IV)-peptide conjugates were also designed and synthesized for the purpose of targeted drug delivery to tumour endothelial cells and tumour cells expressing  $\alpha_v\beta_3/\alpha_v\beta_5$  integrins.<sup>151</sup> The tri- and pentapeptides, containing an RGD (Arg–Gly–Asp) motif, attached by an amide linkage to the platinum(IV) center through a succinato group (Fig. 4), served in this case as tumour-targeting units. Similarly, a Pt(IV) complex conjugated to the cancer-targeting peptide chlorotoxin (CTX) was synthesized in order to deliver the compound selectively to cancer cells.<sup>152</sup> Like most Pt(IV) derivatives, the cytotoxicity of the conjugate was lower in cell culture than that of cisplatin, but greater than those of its Pt(IV) precursor and CTX in several cancer cell lines. Within this frame, application of carbohydrate–Pt complexes is another



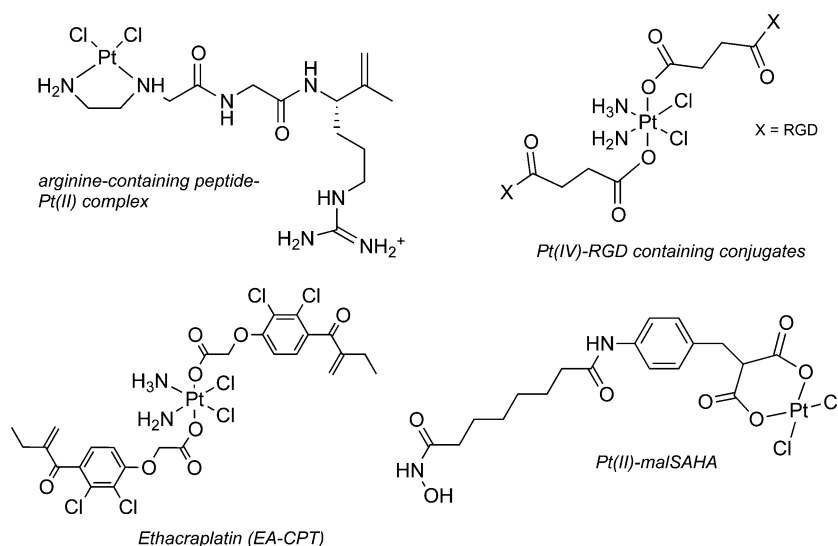


Fig. 4 Examples of Pt complexes designed for targeted therapies.

example of a targeted approach exploiting the biochemical and metabolic functions of diverse sugars in living organisms for transport and accumulation.<sup>153</sup>

In other cases, Pt complexes with ligands acting in synergy with the metal centre to inhibit specific enzymes have been developed (Fig. 4).<sup>154</sup> Thus, diethyl[(methylsulfinyl)methyl]phosphonate (SMP) ligands have been proposed to achieve bone tissue specificity, and Pt-SMP compounds were designed in such a way that the DNA-damaging property is associated with a non-competitive inhibitory effect toward matrix metalloproteinase (MMP) enzymes.<sup>155</sup> Ethacraplatin (EA-CPT), a *trans*-Pt(IV) carboxylate complex containing ethacrynic acid ligands (see Fig. 4), was designed as a platinum anticancer metallodrug that could also target cytosolic glutathione S-transferase (GST P1-1) enzymes.<sup>156</sup> Cytosolic GST enzymes constitute the main cellular defence against xenobiotics, and they are known to catalyze the conjugation of glutathione (GS-H) with cisplatin *in vitro*,<sup>157</sup> the first step in the mercapturic acid pathway that leads to elimination of toxic compounds. The same authors reported that EA-CPT was an excellent inhibitor of GST activity in mammalian cells compared to either cisplatin or ethacrynic acid.<sup>158</sup> Following this trend, Marmion *et al.* developed bifunctional platinum(II) compounds with dual DNA binding and histone deacetylase (HDAC) inhibitory activity of the type reported in Fig. 4 (the Pt(II)-malSAHA compound).<sup>159,160</sup> Finally, terpyridine-Pt(II) complexes have been shown to be effective inhibitors of mammalian topoisomerase enzymes,<sup>161</sup> as well as of the selenoenzyme human thioredoxin reductase 1 (hTrxR1);<sup>162</sup> both targets offering possible novel strategies for future generations of chemotherapeutic agents. Overall, in designing new Pt anticancer drugs, it is now clear that considering only DNA-binding properties are not sufficient.

## 6. Concluding remarks/summary and outlook

Platinum-based anti-cancer drugs are effective pharmaceuticals and are still the most used agents against malignancies. In parallel to the preparation and screening of platinum complexes

as potential anticancer agents, extensive efforts must be directed at elucidating the mechanism of action of platinum-based drugs. Numerous experimental evidences demonstrate that reactions with proteins are fundamental in determining the overall pharmacological and toxicological profile of platinum drugs. Certainly, it is impossible to predict the reactivity of metallodrugs with proteins or peptides *in vivo* only on the basis of classical coordination experiments in aqueous solution. Only an understanding of the cellular events that take place as a result of exposure of cells/tissues to platinum drugs might lead to new strategies for the preparation of novel platinum compounds with improved therapeutic profiles.

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